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Kojic Acid Production by *Aspergillus flavus* Using Gelatinized and Hydrolyzed Sago Starch as Carbon Sources

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ABSTRACT. Direct conversion of gelatinized sago starch into kojic acid by *Aspergillus flavus* strain having amylolytic enzymes was carried out at two different scales of submerged batch fermentation in a 250-mL shake flask and in a 50-L stirred-tank fermentor. For comparison, fermentations were also carried out using glucose and glucose hydrolyzate from enzymic hydrolysis of sago starch as carbon sources. During kojic acid fermentation of starch, starch was first hydrolyzed to glucose by the action of α-amylase and glucoamylase during active growth phase. The glucose remaining during the production phase (non-growing phase) was then converted to kojic acid. Kojic acid production (23.5 g/L) using 100 g/L sago starch in a shake flask was comparable to fermentation of glucose (31.5 g/L) and glucose hydrolyzate (27.9 g/L) but in the 50-L fermentor was greatly reduced due to non-optimal aeration conditions. Kojic acid production using glucose was higher in the 50-L fermentor than in the shake flask.

Kojic acid has several applications in pharmaceutical industry (Kayahara *et al.* 1990), in food industry (Le Blanch and Akers 1989) and in cosmetics (Ohyama and Mishima 1990). It can be produced in submerged batch fermentation (Kitada *et al.* 1967; Ariff *et al.* 1996) or resuspended cell material in solution containing only glucose (Bajpai *et al.* 1982; Ariff *et al.* 1997) by a variety of microorganisms, such as *Aspergillus* and *Penicillium* species. Numerous substances of different molecular size have been utilized effectively as substrates for kojic acid production, such as ethanol, acetate, shikimate, quinate, sucrose and fructose (Beelik 1956). Although many carbon sources can be used for kojic acid production, glucose is known as the best cabon source due to the similarity of its structure to that of kojic acid. It has been suggested that kojic acid is formed directly from glucose without any cleavage of the carbon chain into smaller fragments (Arnstein and Bentley 1953). On the other hand, polysaccharides (*e.g.* starch) are poor carbon sources for kojic acid production (Kitada *et al.* 1967). The use of starch, which is an inexpensive carbon source, will reduce the cost of raw materials for the production. Recently, a kojic acid-producing fungus, *Aspergillus flavus* strain 44-1, capable of using starch as substrate, has been isolated (Madihah 1996). When starch is used as carbon source, kojic acid produced by *A. flavus* has a higher solubility and cytotoxicity than that produced by fermentation using glucose (Hwei 1996). However, the efficiency of this fungus to produce kojic acid from sago starch on different scales and techniques of fermentation has not yet been reported.

The present work was undertaken to investigate the feasibility of using sago starch and glucose hydrolyzate from enzymic hydrolysis of sago starch as carbon sources for kojic acid production. Submerged batch fermentation of *A. flavus* was carried out at two different scales of production, a 250-mL shake flask and a 50-L stirred tank fermentor. Production was also carried out in a resuspended cell system using a shake flask.

MATERIALS AND METHODS

Microorganism and medium. Aspergillus flavus strain 44-1, isolated at the *Department of Biotechnology, Universiti Putra Malaysia*, was used for kojic acid production. The optimized medium for kojic acid production proposed by Madihah (1996) was used, though different types of carbon sources (glucose, glucose hydrolyzate and gelatinized starch) were added according to the needs of each experiment. Sago starch used in this study was supplied by *Nee Seng Le Co.*, Sarawak (Malaysia). Gelatinized starch was prepared by heating starch slurry to slightly above 70 °C, which is the gelatinization temperature of sago starch (Haska and Ohta 1992). Sago starch was hydrolyzed enzymically to glucose hydrolyzate (Arbakariya *et al.* 1990). The hydrolysis involved two processes, liquefaction and

saccharification by using α-amylase (Termamyl 120) and glucoamylase (Dextrozyme 225/70L), respectively. Both enzymes were obtained from *NOVO*, Kuala Lumpur (Malaysia).

Submerged fermentations. Batch fermentations of kojic acid were carried out using a shake flask and a 50-L Biostat-U stirred tank fermentor (*B. Braun*, Melsungen, Germany). The fermentor was equipped with temperature and dissolved-oxygen controllers. Three six-blade turbine impellers (diameter d = 125 mm) were used for agitation and the agitation speed (n) was fixed at 4.2 Hz (impeller tip speed = $2\pi nd$ = 3.29 m/s).

In all fermentations, spore suspension containing about 10^5 spores per mL medium was used as inoculum. The 250-mL shake flask containing 100 mL medium with initial pH 3.0 was inoculated and incubated at 30 °C in a rotary shaker agitated at 4.2 Hz. During fermentation in the fermentor, temperature was maintained at 30 °C under aeration control strategy for optimum kojic acid production as suggested previously (Ariff *et al.* 1996) was employed. The initial culture pH was adjusted to 3.0 and the pH was not controlled during fermentation. All fermentations were carried out at least in duplicate.

Resuspended cell system. The production of cell material was first carried out in a shake flask using the same medium and cultural conditions as in batch submerged fermentation. After growth reached a stationary phase (after 5 d of cultivation), cells were harvested by centrifuging (6 000 *g*, 20 min) and then washed with sterile deionized water and recentrifuged. The pellets were then resuspended in a shake flask containing 100 mL of glucose or glucose hydrolyzate (100 g/L) in sterile citrate buffer (pH 3.0). The flasks were incubated at 30 °C in a rotary shaker agitated at 4.2 Hz.

Analytical methods. Samples of culture broth were filtered using preweighed microfiber filter paper. The supernatants were used for kojic acid and other chemical determinations while the residues were dried in an oven at 95 °C for dry matter determination. Kojic acid was analyzed using HPLC (Ariff *et al.* 1996). Glucose concentration was determined enzymically using *Sigma Diagnostic Glucose Trinder* reagent. Glucoamylase was assayed using the method of Ariff and Webb (1996) and α-amylase was assayed according to Bhella and Altosaar (1984).

RESULTS AND DISCUSSION

Kojic acid fermentation in a shake flask. The typical time course of kojic acid fermentation in a shake flask using glucose hydrolyzate, glucose, 100 or 50 g/L gelatinized sago starch as carbon sources is shown in Fig. 1. The fungus grew well on all types of carbon source; growth was very rapid during the initial stages of fermentation. When glucose hydrolyzate and glucose were used as carbon sources, stationary growth phase was reached after about 5 d. On the other hand, stationary phase was only achieved after about 9 d of fermentation of sago starch. The changes in the external medium with different types of carbon sources greatly affected the growth morphology of the fungus. When glucose hydrolyzate and gelatinized starch were used, cells grew in the form of mycelia. While in glucose cells grew in the form of spherical pellets with an average diameter of 0.5–1 mm.

During fermentation of sago starch, glucose concentration rose rapidly and the highest concentration was obtained after about 4 d (Fig. 1C, D). The highest glucose concentrations obtained in the culture during fermentation of 100 and 50 g/L sago starch were 50.1 and 25.2 g/L, respectively. *A. flavus* was capable to hydrolyze starch to glucose by secreting substantially higher activities of amylolytic enzymes, such as α-amylase and glucoamylase. The patterns of α-amylase and glucoamylase production followed the pattern of cell growth, indicating that the processes were associated. However, both enzyme activities decreased when growth reached a stationary phase. In all fermentations, rapid kojic acid production occurred during the nongrowing phase where rapid consumption of glucose was also observed, suggesting that glucose was used for kojic acid synthesis. Kojic acid production ceased when glucose in the culture became depleted.

Comparison of kojic acid fermentation using different types of carbon source is given in Table I. The maximum cell concentration obtained in fermentations of sago starch was slightly higher than those obtained in fermentations of glucose and glucose hydrolyzate. The highest kojic acid production was obtained from fermentation of glucose, which was about 14 % higher compared to fermentation of glucose hydrolyzate. Kojic acid production in fermentation of 100 g/L sago starch was comparable with fermentation of glucose hydrolyzate. However, low kojic acid production (12.4 g/L) was obtained from fermentation of 50 g/L sago starch. Reduced production in fermentation of 50 g/L starch may be due to low concentration of glucose remaining in the culture during the nongrowing phase for kojic acid synthesis. The amounts of glucose remaining in the culture during the nongrowing phase of fermentation using 100 and 50 g/L starch were 35.0 and 10.0 g/L, respectively. This means

that about 80 % glucose produced in fermentation of 50 g/L starch was used for growth and only about 20 % for conversion to kojic acid.

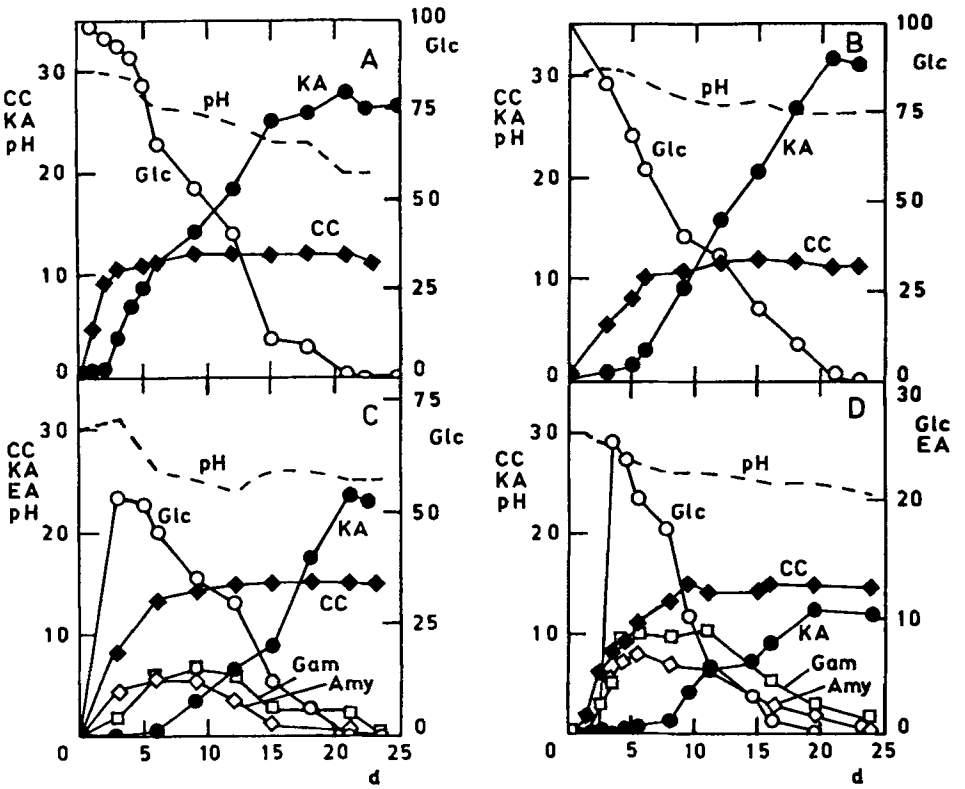

Fig. 1. Time courses of batch kojic acid fermentation by *A. flavus* in a shake flask using glucose hydrolyzate (**A**), glucose (**B**), 100 g/L sago starch (**C**) and 50 g/L sago starch (**D**); CC — cell concentration (g/L), KA — kojic acid (g/L), Glc — glucose (g/L), Amy — α-amylase (EA, U/mL), Gam — glucoamylase (EA, U/mL), pH (×10).

Table I. Comparison of kojic acid fermentation by *A. flavus* in a shake flask and in a 50-L fermentor on different types of carbon source

Parameter[a]	Shake flask				50-L fermentor			
	Glucose hydrolyzate 100 g/L	Glucose 100 g/L	Gelatinized starch 100 g/L	Gelatinized starch 50 g/L	Glucose hydrolyzate 100 g/L	Glucose 100 g/L	Gelatinized starch 100 g/L	Gelatinized starch 50 g/L
X_m, g/L	12.1	11.8	15.1	15.0	12.8	12.7	14.2	13.6
P_m, g/L	27.9	31.5	23.5	12.4	28.8	49.7	0.2	14.8
$Y_{p/s}$, mg/g	279	315	235	247	288	497	2.13	296
t, h	504	516	504	498	282	234	231	225
P, mg L^{-1} h^{-1}	55	61	47	25	102	213	0.92	65.8

[a] X_m — maximum cell concentration obtained during fermentation, P_m — maximum kojic acid concentration, t — time taken to reach maximum concentration of kojic acid, $Y_{p/s}$ — yield of kojic acid based on carbon source consumed, P — productivity.

Kojic acid fermentation in a 50-L stirred tank fermentor. Fig. 2 shows the time course of kojic acid production in a 50-L stirred tank fermentor using different types of carbon source. Similar to fermentation in a shake flask, growth of *A. flavus* in starch was slower compared to glucose. In fermentations of sago starch, stationary growth phase was reached after about 10 d of cultivation. While stationary growth phase for fermentations of glucose and glucose hydrolyzate was achieved after about 4 d. In fermentations of glucose and glucose hydrolyzate, cells grew in the form of a pellet. On the other hand, cells grew in the form of mycelia during fermentation of starch. This result indicates that the growth

morphology was not only affected by the type of carbon source but also the different in hydrodynamic conditions and shear rates created in a shake flask and the fermentor.

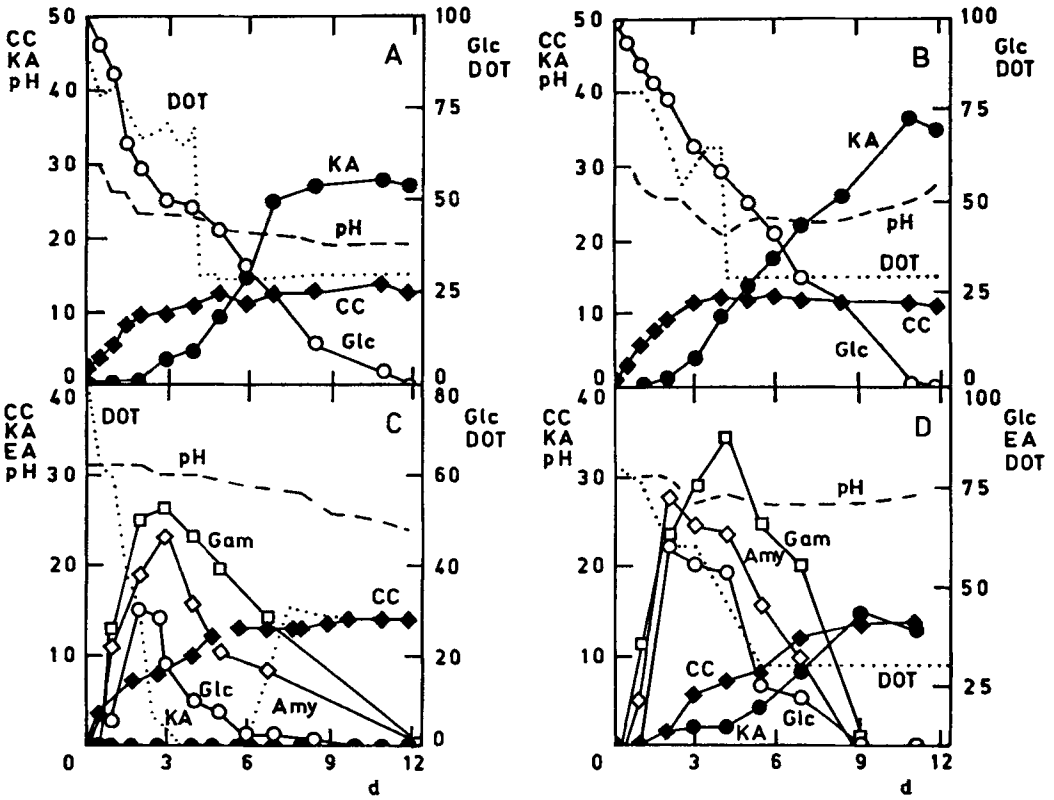

Fig. 2. Batch kojic acid fermentation by *A. flavus* in a 50-L fermentor using glucose hydrolyzate (A), glucose (B), 100 g/L sago starch (C) and 50 g/L sago starch (D); CC — cell concentration (g/L), KA — kojic acid (g/L), Glc — glucose (g/L), Amy — α-amylase (EA, U/mL), Gam — glucoamylase (EA, U/mL), pH (×10), DOT (% saturation).

Although growth was excellent in fermentation of sago starch, kojic acid production was very low (0.2 g/L). Reduced kojic acid production in fermentation of 100 g/L starch using the 50-L fermentor may be due to nonoptimal aeration conditions. During active growth, dissolved oxygen tension (DOT) in the culture decreased rapidly and the level became zero after 80 h and only increased to 30 % saturation after 7.5 d of fermentation. High viscosity of starch during the initial stages of fermentation resulted in imperfect mixing and a low oxygen transfer rate so the optimum aeration conditions for kojic acid production as described previously (Ariff *et al.* 1996) were not achieved. For maximum production of kojic acid by *A. flavus*, the DOT during growth and production phases should be maintained at 80 and 30 % saturation, respectively. The cell-bound enzyme system consisted of enzymes, such as glucose-6-phosphate dehydrogenase, hexokinase and gluconate dehydrogenase, involved in the direct synthesis of kojic acid from glucose (Bajpai *et al.* 1981). High DOT levels during active growth were required for enhancement of the synthesis of these enzymes (Ariff *et al.* 1996). In addition, oxygen-limited conditions during the growth phase also caused reduction of amylolytic enzyme production, which in turn reduced the production of glucose in the culture. This may be another reason for low kojic acid production in fermentation of high starch concentrations. Because an optimal aeration condition was achieved during fermentation of 50 g/L starch, higher levels of kojic acid were produced than in fermentation of 100 g/L starch. Although optimum aeration conditions during fermentation of glucose and glucose hydrolyzate obtained, kojic acid production from fermentation of glucose hydrolyzate (28.8 g/L) was about 50 % of that obtained from fermentation of glucose (49.7 g/L). Some impurity present in glucose hydrolyzate may inhibit kojic acid production.

Comparison of the performance of kojic acid production between fermentation in a shake flask and in the 50-L fermentor using different types of carbon source is also summarized in Table I. There was no significant difference in maximum cell concentration (X_m) obtained from fermentations

using different types of carbon source in shake flask and 50-L fermentor. However, kojic acid production was significantly different at different scales of production. Kojic acid production in fermentation of glucose and glucose hydrolyzate was significantly higher in the 50-L fermentor than in a 250-mL shake flask. On the other hand, kojic acid production from fermentation of sago starch in a 50-L fermentor was greatly reduced compared to fermentation in the shake flask. It is worth to note that the fermentation time for kojic acid production in the fermentor was reduced about two times shorter than those required in shake flask fermentation. Hence, an increased overall productivity was observed. The reason for the changes in production at different scales of fermentation seemed to be due to different DOT profiles during fermentation. Production of *A. flavus* mycelia with a great ability for synthesizing kojic acid can be achieved either by oxygen limitation or high DOT levels (>70 % saturation) during the growth phase (Ariff *et al.* 1996). Fermentation in a shake flask may be subject to oxygen limitation; thus substantially higher kojic acid was produced with a longer period of fermentation because growth rate was decreased. By controlling DOT at high levels during active growth in a 50-L fermentor, not only mycelia with high ability to synthesize kojic acid was produced, but the growth phase period was also reduced.

Kojic acid production in resuspended-cell system. The enzyme system involved in kojic acid biosynthetic pathway was found to be very stable for a long starvation period (Bajpai *et al.* 1982; Ariff *et al.* 1997). The efficient utilization of the remaining activities from batch fermentation might be due to the extension of the production phase with glucose feeding alone or in a resuspended-cell system. Fig. 3 shows the time courses of kojic acid production in a resuspended-cell system using glucose hydrolyzate and glucose. Cell concentrations remained constant after resuspended in glucose hydrolyzate or glucose, indicating that glucose was not utilized for cell growth but predominantly for the conversion to kojic acid. In the resuspended-cell system, kojic acid production was higher in glucose (39.2 g/L) compared to glucose hydrolyzate (22.6 g/L). When cell material was resuspended in gelatinized sago starch, glucose and kojic acid production were not observed (*data not shown*). This result indicates that amylolytic enzymes were not secreted during non-growing phase of *A. flavus* and starch cannot be converted to kojic acid by the enzyme reaction during the production phase.

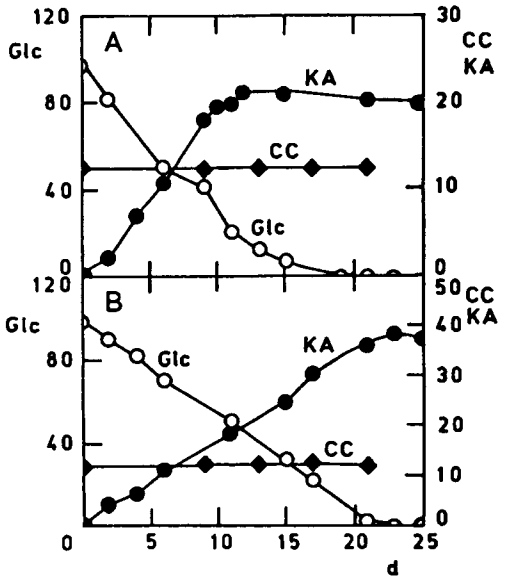

Fig. 3. Kojic acid production in resuspended cell system using glucose hydrolyzate (A) and glucose (B); CC — cell concentration (g/L), KA — kojic acid (g/L), Glc — glucose (g/L).

Gelatinized sago starch can be utilized by *A. flavus* as a carbon source in batch submerged kojic acid fermentation using a 250-mL shake flask. Starch was first hydrolyzed to glucose by the amylolytic enzymes (α-amylase and glucoamylase) secreted by the fungus during active growth. During the production phase (nongrowing phase), the remaining glucose in the culture was then converted to kojic acid. Scaling-up the fermentation of 100 g/L sago starch resulted in almost no production of kojic acid. However, kojic acid production from fermentation of 50 g/L sago starch in a 50-L fermentor was comparable to that obtained from fermentation in a shake flask. Insufficient oxygen supply during the fermentation of high starch concentrations was one of the possible reasons for reduced kojic acid production. With optimum aeration control strategy, kojic acid production using glucose and glucose hydrolyzate in a 50-L fermentor was higher than those obtained in a shake flask. Further work is being carried out in our laboratory to find the optimum fermentor aeration conditions which are required for increasing kojic acid production using high-viscosity gelatinized sago starch in a large-scale fermentor.

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